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Circadian regulation of low density lipoprotein receptor promoter activity by CLOCK/BMAL1, Hes1 and Hes6

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Abbreviations: BMAL1, brain and muscle arnt-like 1; CLOCK, circadian locomotor output cycle kaput; CRY1, cryptochrome 1; Hes1, hairy and enhancer of split 1; Hes6, hairy and enhancer of split 6; IDL, intermediate density lipoprotein; LDLR, low density lipoprotein receptor; PER1, period homolog 1; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; SRE, sterol regulatory element

Abstract

Low density lipoprotein receptor (LDLR) plays an important role in the cholesterol homeostasis. We examined the possible circadian regulation of LDLR and mechanism(s) underlying it. In mice, blood glucose and plasma triglyceride, total and high density lipoprotein cholesterol varied distinctively throughout a day. In addition, LDLR mRNA oscillated in the liver in a functional clock-dependent manner. Accordingly, analysis of human LDLR promoter sequence revealed three putative E-boxes, raising the possible regulation of LDLR expression by E-box-binding transcription factors. To test this possibility, human LDLR promoter reporter constructs were transfected into HepG2 cells and the effects of CLOCK/BMAL1, Hes1, and Hes6 expression were analyzed. It was found that positive circadian transcription factor complex CLOCK/BMAL1 upregulated human LDLR promoter activity in a serum-independent manner, while Hes family members Hes1 and Hes6 downregulated it only under serum-depleted conditions. Both effects were mapped to proximal promoter region of human LDLR, where mutation or deletion of well-known sterol regulatory element (SRE) abolished only the repressive effect of Hes1. Interestingly, *hes6* and *hes1* mRNA oscillated in an anti-phasic manner in the wild-type but not in the *per1*$^{-/-}$*per2*$^{-/-}$ mouse. Comparative analysis of mouse, rat and human *hes6* genes revealed that three E-boxes are conserved among three species. Transfection and site-directed mutagenesis studies with *hes6* reporter constructs confirmed that the third E-box in the exon IV is functionally induced by CLOCK/BMAL1. Taken together, these results suggest that LDLR expression is under circadian control involving CLOCK/BMAL1 and Hes family members Hes1 and Hes6.

Keywords: ARNTL transcription factors; circadian rhythm; CLOCK proteins; HES1 protein, human; HES6 protein, human; receptors, LDL

Introduction

The lives of most organisms living on earth are governed by the repetitive changes of day and night generated by rotation of the earth on its axis. The circadian (*circa*: around, *dies*: day) clock enables an organism to anticipate environmental changes throughout a day, to ensure proper timing for biological processes, and to improve the efficiency of biological energy uses. Circadian rhythms govern many aspects of physiological and metabolic functions including the sleep-wake cycle, feeding behavior, body temperature, as well as cardiovascular, endocrine, gastrointestinal, hepatic and renal functions.

In mammals, circadian rhythms are endogenously generated by 'central/master pacemaker' in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, which orchestrates many 'peripheral/slave oscillators' present in numerous peripheral tissues. The central clock is entrained by external time cues called 'Zeitgeber', such as light. In photic entrainment, SCN receives inputs through retinohypothalamic tract (RHT) and synchronizes the peripheral clocks in peripheral tissues by the neural and humoral

outputs (Moore *et al*., 1983; Panda and Hogenesch, 2004; Noh *et al*., 2011). In addition to the light, another important Zeitgeber for the peripheral tissues is food (Stokkan *et al*., 2001; Ishida *et al*., 2005; Yoon *et al*., 2012).

The molecular machinery of circadian clock is composed of complex positive and negative transcriptional and translational feedback loops. The positive clock components, CLOCK and BMAL1 (MOP3) form heterodimer and activate transcription of target genes by binding to the E-box sequence (CANNTG) on their regulatory sequence. The negative components, PERs and CRYs containing E-box on their promoters are induced by CLOCK/BMAL1, accumulate in the cytoplasm, translocate to the nucleus with timed delay, and inhibit the action of CLOCK/BMAL1. This feedback loop generates transcriptional and translational fluctuations and allows various rhythms of enzymes activity, hormone secretion and many physiological aspects with ~24 h period even under constant conditions (Dunlap, 1999; Roenneberg *et al*., 2003).

Dysregulation of circadian rhythms in human may result in pathological conditions. For example, shift workers are subject to unusual daily cycle so that their disruption of biological rhythms leads to disorders in physiology and metabolism. Indeed, many studies have reported increased obesity, type II diabetes, and cardiovascular diseases in shift workers (Knutsson, 2003; Ha *et al*., 2005).

The relation between circadian dysregulation and pathophysiology can be mechanistically studied in mice. By mutation or knockout of core clock components, the role of each clock component in numerous physiological processes can be understood. In these mutated mice, various pathological conditions including metabolic syndrome, abnormal gluconeogenesis, apoptosis/cancer development, abnormal lipogenesis and retinal degeneration/blind has been reported (reviewed in Ko and Takahashi, 2006). For example, *Clock/Clock* mutant mice become obese and develop metabolic syndrome with hepatic steatosis, hyperglycaemia, hypertriglyceridaemia, hypercholesterolaemia and hyperleptinaemia (Turek *et al*., 2005). This reveals that circadian regulation is linked to metabolism and dysregulation of circadian rhythms can contribute to metabolic diseases.

The cholesterol level in blood is related to several metabolic diseases such as hypercholesterolaemia, high blood pressure, atherosclerosis and cardiovascular diseases. According to recent studies (Whitmer *et al*., 2008), it also triggers dementia and decline of renal function. Thus, proper regulation of blood cholesterol is important for health and wellbeing.

Cholesterol is an important component for the manufacturing of bile acids, steroid hormones, cell membrane and several fat-soluble vitamins, and it is either taken *via* dietary sources or synthesized *de novo* within the body. Since cholesterol is insoluble in blood, it is transported in the circulatory system by lipoprotein carriers, such as chylomicrons, very low density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs), low density lipoprotein (LDLs), and high density lipoproteins (HDLs). Among these, LDL is the major carrier of cholesterol in the blood and high levels of LDL-cholesterol in the blood is related to several diseases. LDL molecules can be oxidized and taken up by macrophages, which become engorged and form foam cells. These cells often become trapped in the walls of blood vessels and contribute to atherosclerotic plaque formation. These plaques are one of the main causes of heart attacks, strokes, and other serious medical problems. Thus, proper regulation of LDL-cholesterol within the blood is important for the prevention of atherosclerosis. In various organs including the liver, LDL-cholesterols are uptaken through LDL receptor-mediated endocytosis. LDL receptor (LDLR) is located on the cellular membrane and it recognizes apoB-100 of LDL and carries LDL particles into cells through coated pits. The cholesterol released from LDL can be used or stored, and remaining LDLRs can be either recycled or degraded. It is well known that the synthesis of LDLRs is regulated by SREBP through SRE present on the LDLR promoter (Briggs *et al*., 1993). The cholesterol levels within the blood throughout the day remain stable regardless of feeding or fasting. However, the well-defined diurnal rhythm of HMG-CoA reductase (Edwards *et al*., 1972) raises the possibility of circadian expression of LDLR. Indeed, it was once reported that LDLR protein activity displays daily fluctuations in the rat (Balasubramaniam *et al*., 1994). The purpose of this study is to examine possible circadian regulation of LDLR gene expression and its molecular mechanism(s).

Results

Daily rhythms of metabolic parameters in mice fed *ad libitum*

Nocturnal mice consume most of their food during the early scotophase when fed *ad libitum* (Sanchez-Alavez *et al*., 2007). To see whether mice fed *ad libitum* display daily variation in the metabolic parameters related to cholesterol homeostasis, daily changes in the levels of blood glucose, triglyceride (TG), total cholesterol and HDL cholesterol were determined (Figure 1). Blood glucose levels varied throughout a day, reaching the lowest level in the

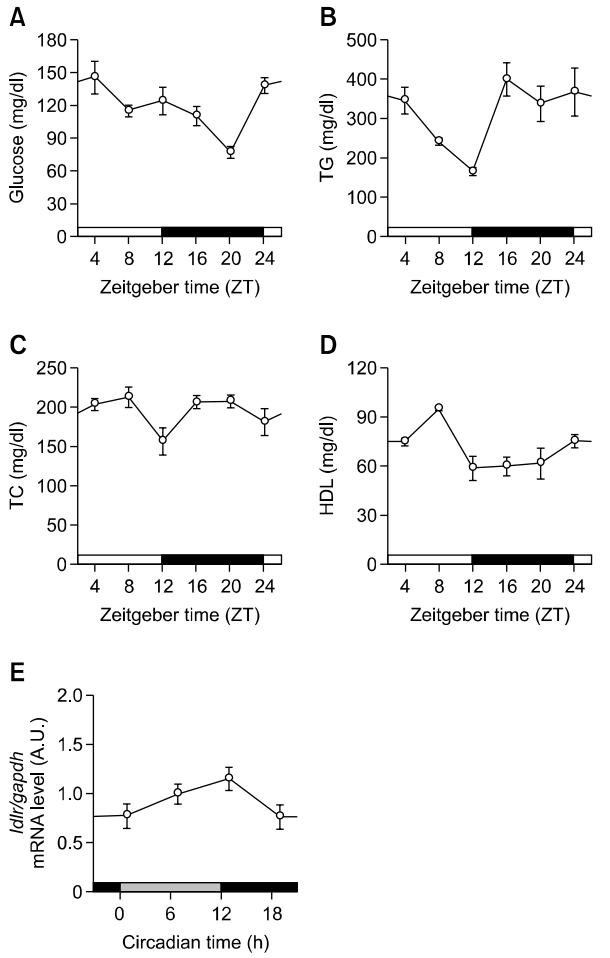

Figure 1. Daily rhythms of metabolic parameters related to cholesterol homeostasis in mice fed *ad libitum*. (A-D) Male C57BL/6 mice were entrained to 12L:12D cycle for 2 weeks and sacrificed by cervical dislocation at the indicated zeitgeber time (ZT) and whole blood samples were collected. Blood glucose (A), plasma TG (B), total cholesterol (C) and HDL cholesterol were determined by specific kits obtained from Callegrari™. Data are expressed as mean ± S.E.M. (n = 8). (E) Oscillation of *Idlr* mRNA in the mouse liver. Male C57BL/6 mice were entrained to 12L:12D cycle for 2 weeks and released to DD. On the second day after light-off, mice were sacrificed by cervical dislocation at the indicated circadian time and liver samples were obtained. RNA isolation, reverse transcription, and real-time polymerase chain reaction were performed to measure specific messages for mouse *Idlr*. All mRNA levels were normalized to *gapdh* mRNA level and expressed as mean ± S.E.M. (n = 8).

middle of active/dark phase (ZT20; Figure 1A). TG and HDL cholesterol levels also displayed distinct daily patterns. TG levels remained high throughout the active phase but declined during the rest reaching lowest level at ZT12 (Figure 1B). Total cholesterol levels remained fairly stable throughout a day but decreased at the time of light-dark transition (Figure 1C). HDL cholesterol remained low during the dark phase but increased during the light phase reaching highest level at ZT8 (Figure 1D). These data indicate that metabolic parameters related to cholesterol homeostasis vary throughout the day in mice fed *ad libitum*. To see whether *Idlr* mRNA oscillates in a circadian manner, mouse liver samples were obtained and analyzed every 6 h (Figure 1E). In the mouse liver, mouse *Idlr* mRNA displayed circadian rhythm of gene expression having acrophase at around CT12. This is in good agreement with the previous report that LDLR protein reach the highest level at ZT12 in the rat (Balasubramaniam *et al*., 1994).

Serum-independent induction by CLOCK/BMAL1 and serum-dependent repression by Hes1 and Hes6 of the human *Idlr* promoter activity

To investigate the transcriptional regulation of human *Idlr*, promoter sequences were analyzed. Schematic diagrams of DNA elements on human *Idlr* gene promoter and serial deletion reporter constructs are presented in Figure 2. The sterol regulatory element (SRE) is located in the proximal region and three putative E-boxes are located within 1 kb of the upstream promoter. Serial deletion constructs (Jeong *et al*., 2009) were used to examine responsiveness to CLOCK/BMAL1, Hes1, and Hes6. These constructs were transiently transfected into HepG2 human hepatocarcinoma cells with or without CLOCK/BMAL1, Hes1, or Hes6 expression vectors. Human *Idlr* gene expression is induced by CLOCK/BMAL1 in serum-independent manner. However, the inhibitory effects of Hes1 and Hes6 were observed only under serum-depleted condition. In the presence of serum, Hes1 and Hes6 showed no effect. These results indicate that transcriptional regulation of *Idlr* gene is under circadian control involving CLOCK/BMAL1 and Hes family transcription factors Hes1 and Hes6. To see whether the mouse *Idlr* gene is also regulated in the same manner, we compared the promoter sequences between mouse and human *Idlr* genes and generated mouse *Idlr* promoter constructs as shown in Supplemental Data Figure S1. We found that SRE elements important for the inhibitory effect of Hes1 and Hes6 are well conserved between human and mouse *Idlr* genes, while several E-boxes found in distal regions of human and mouse promoters are not so well conserved. Then, we tested whether mouse *Idlr* gene is also regulated by CLOCK/BMAL1, Hes1 and Hes6 (Supplemental Data Figure S2). We found that mouse *Idlr* transcription is also reduced by Hes1 and Hes6 expression, while the induction by CLOCK/BMAL1 was quite weaker compared with the induction of human constructs (compare Figure S2 with Figures 2B and 2C), which is in good agreement with the slight increase of mouse LDLR

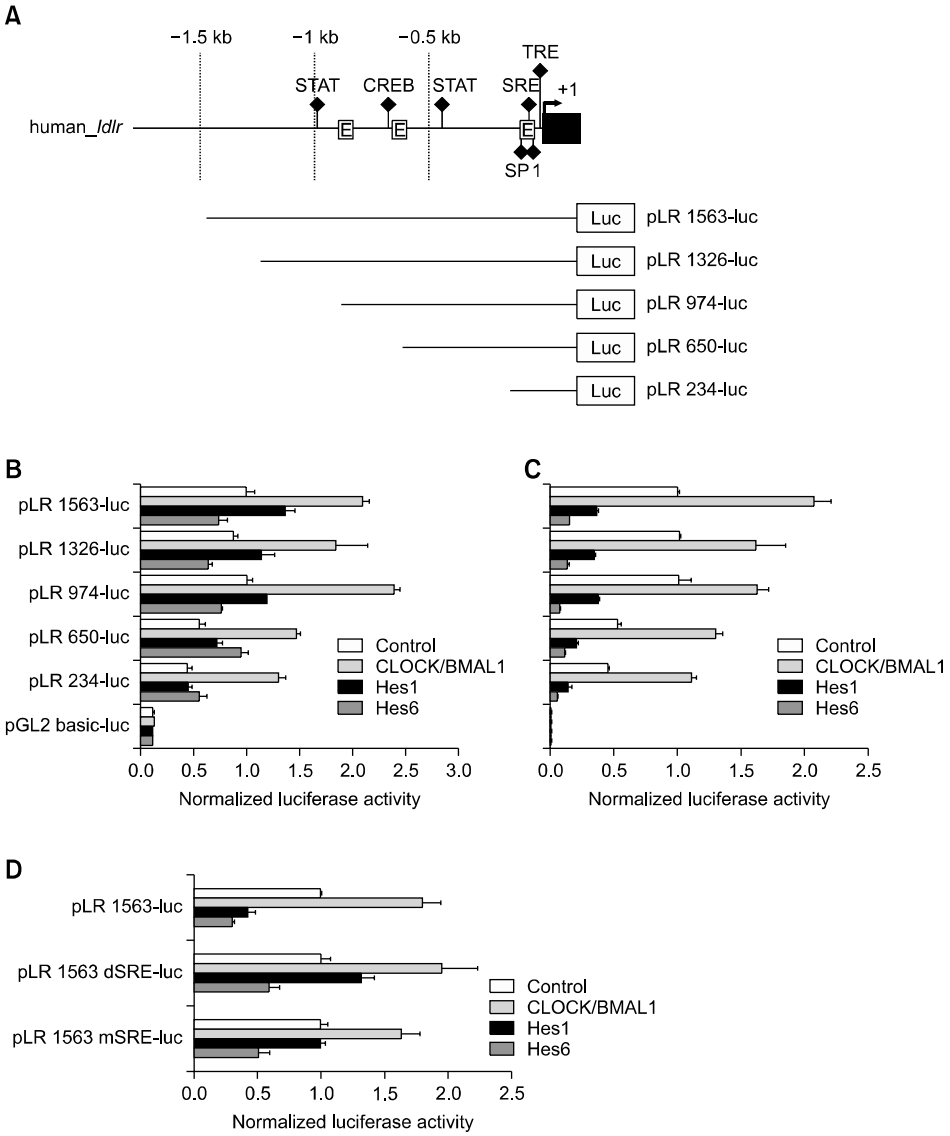

Figure 2. Transcriptional regulation of *ldlr* promoter activity by CLOCK/BMAL1, Hes1 and Hes6. (A) Schematic diagram of DNA elements on human *ldlr* gene promoter and serial deletion constructs. Genomic DNA sequences spanning human *ldlr* (NT_011295, chromosome 19) were analyzed for the transcription factor binding sites using tools provided by Genomatrix (www.genomatrix.de). DNA elements were symbolized as indicated in the figure. The predicted transcription start site is indicated as a bent arrow. Schematic drawing of human *ldlr* gene reporter constructs is also shown in the lower part of the figure. (B, C) Effect of CLOCK/BMAL1, Hes1 and Hes6 expression on reporter activity. HepG2 human hepatocarcinoma cells were transiently transfected with each reporter construct with or without CLOCK/BMAL1, Hes1, or Hes6 expression vectors. One day after transfection, cells received either 10% FBS-containing (B) or serum-depleted media (C). Luciferase assay was performed after additional 24 h incubation. Resulting firefly luciferase activity was normalized to *Renilla* luciferase activity driven by TK promoter, which was used as an internal control. Data are expressed as mean ± S.E.M. (n = 3). (D) The sterol regulatory element (SRE) in the proximal promoter of human *ldlr* gene is responsible for inhibition by HES1. Effect of CLOCK/BMAL1, Hes1 and Hes6 on reporter activity. HepG2 cells were transiently transfected with pLR 1563-luc or pLR 1563 mSRE-luc or pLR 1563 dSRE-luc construct with or without CLOCK/BMAL1, Hes1 and Hes6 expression vectors. Resulting firefly luciferase activity was normalized to *Renilla* luciferase activity driven by TK promoter, which were used as an internal control. Data are expressed as mean ± S.E.M.

mRNA around CT12 (Figure 1E). Moreover, the induction by CLOCK/BMAL1 was blunted with the proximal promoter construct (-263 mldlr-Luc), while the repression by Hes1 and Hes6 still remained. Surprisingly, in contrast to human promoters (Figures 2B and 2C), reduction of mouse *ldlr* transcription by Hes1 and Hes6 were observed also in serum-enriched condition. Thus, there exist some convergent and divergent controls of *ldlr* gene transcriptions between human and mouse promoters.

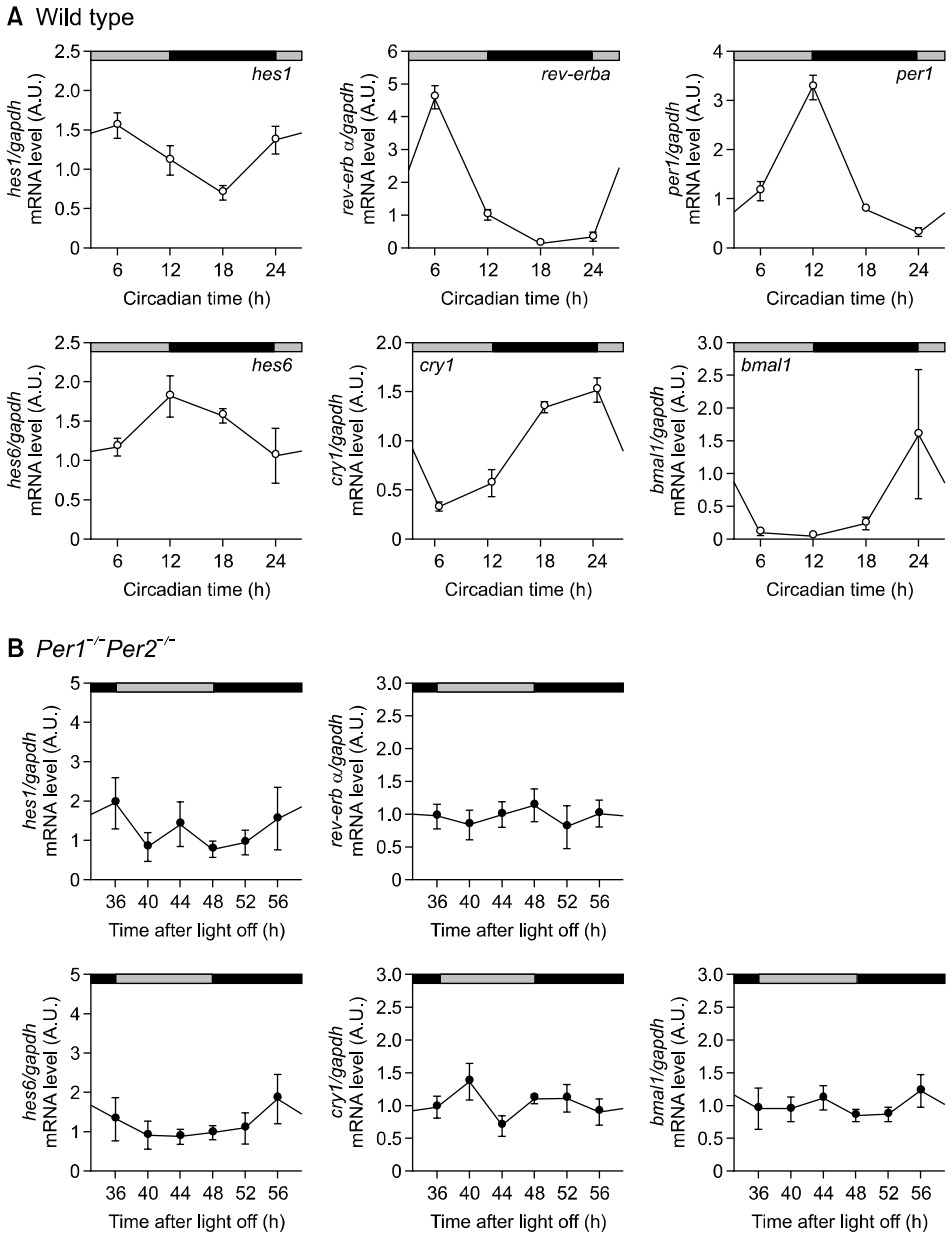

Figure 3. Anti-phasic oscillation of *hes1* and *hes6* mRNA in the wild-type (A) but not in *per1*$^{-/-}$*per2*$^{-/-}$ (B) mouse liver. Male C57BL/6 mice of each genotype were entrained to 12L:12D cycle for 2 weeks and released to DD. On the second day after light-off, mice were sacrificed by cervical dislocation at the indicated circadian time and liver samples were obtained. RNA isolation, reverse transcription, and real-time polymerase chain reaction were performed to measure specific messages for *rev-erba, cry1, bmal1b, hes6* and *hes1*. All mRNA levels were normalized to *gapdh* mRNA level and expressed as mean ± S.E.M. (n = 3).

The sterol regulatory element (SRE) mediates the Hes1-induced transcriptional repression

The serum-dependent inhibitory effect of Hes1 and Hes6 suggests involvement of SRE. To test this possibility, SRE-mutated (pLR 1563 mSRE-luc) or SRE-deleted (pLR 1563 dSRE-luc) *ldlr* reporter constructs were used. These constructs were transiently transfected into HepG2 cells with or without CLOCK/BMAL1, Hes1, or Hes6 expression vectors under serum-depleted condition (Figure 2D). Interestingly, inhibitory effect of Hes1 was abolished but still induced by CLOCK/BMAL1 and repressed by Hes6. These results indicate that Hes1-induced transcriptional repression is mediated by SRE and the Hes6-induced transcriptional repression may involve other element(s).

Anti-phasic oscillation of *hes6* and *hes1* genes expression in the mouse liver

To see whether *hes6* and *hes1* gene expressions are also controlled by circadian clock, *hes6*, *hes1* and various clock genes expressions were analyzed in both WT and circadian clock mutant mice. Wild type and *per1*$^{-/-}$*per2*$^{-/-}$ mice (Park *et al*., 2012) were first entrained to a 12L:12D lighting cycle for 2

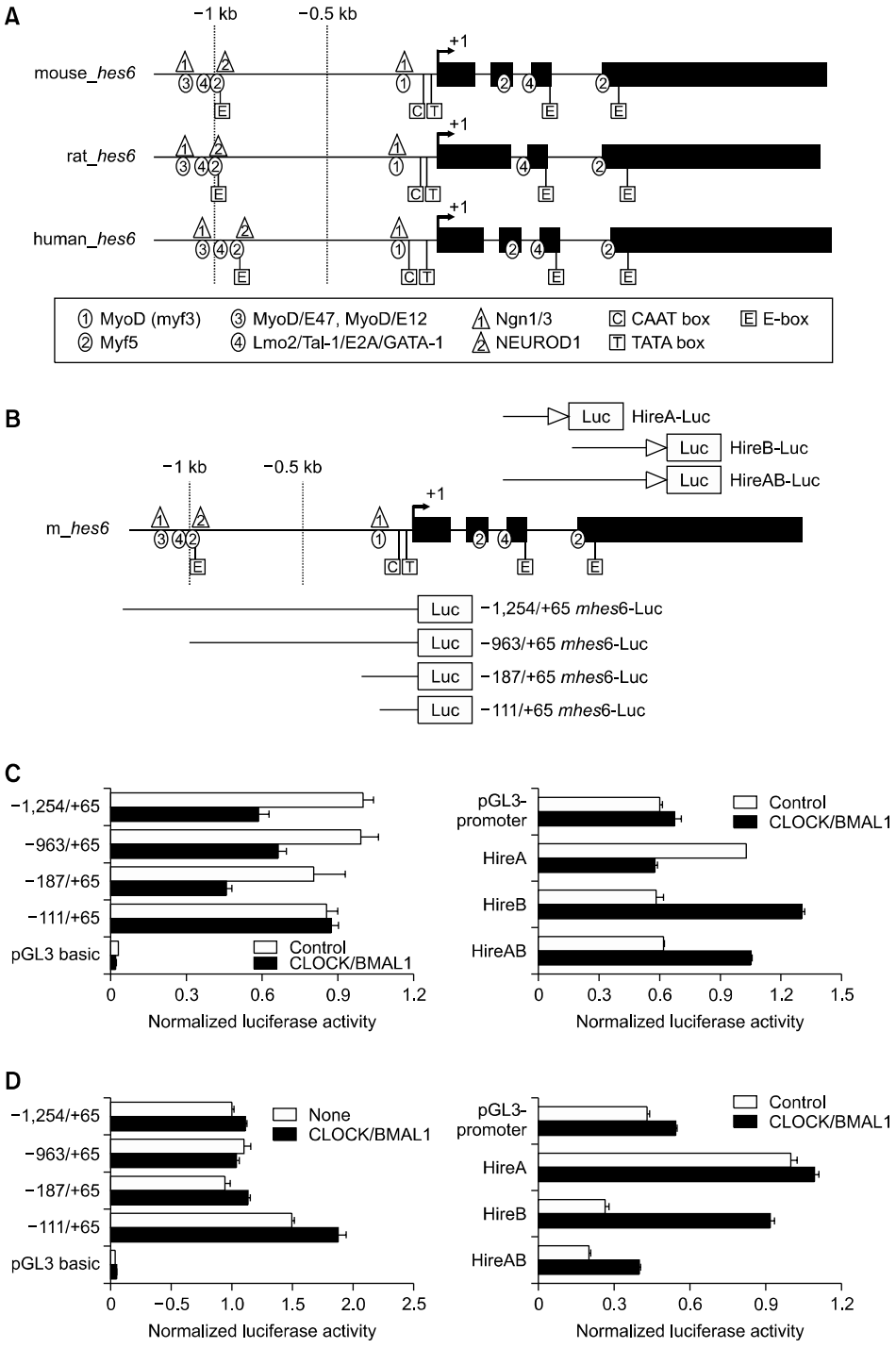

Figure 4. Mouse *hes6* gene is induced by CLOCK/BMAL1 *via* downstream regulatory sequence. (A) Schematic diagrams of mouse, rat, and human *hes6* gene structure and conserved DNA elements. Genomic DNA sequences spanning mouse (Accession No. NT078297, chromosome 1), rat (NW_047817, chromosome 9), and human *hes6* (NT_005120, chromosome 2) were analyzed for the common transcription factor binding sites using tools provided by Genomatrix (www.genomatrix.de). Evolutionarily conserved DNA elements were symbolized as indicated in the figure. (B) Schematic drawing of mouse *hes6* gene reporter constructs. The four serial deletion constructs were made from upstream promoter region of hes6 and regulatory elements were made from intergenic region. See Materials and Methods for details. The right headed triangle indicates SV40 promoter. (C, D) Effect of CLOCK/BMAL1 expression on reporter activity. NIH 3T3 mouse fibroblast (C) and HT22 mouse hippocampal cells (D) were transiently transfected with each reporter construct with or without CLOCK/BMAL1 expression vectors. Resulting firefly luciferase activity was normalized to *Renilla* luciferase activity driven by TK promoter, which was used as an internal control. Data are expressed as mean ± S.E.M.

weeks and released to constant darkness (DD). On the second day after light-off, mouse liver samples were obtained every 6 h from CT6. The mRNA levels of the liver samples were analyzed using real-time RT-PCR (Figure 3). The core clock, *rev-erba*, *per1*, *cry1*, and *bmal1b*, displayed clear circadian rhythm of gene expression having acrophases at around CT6, CT12, CT18-24, and CT24, respectively. The *hes6* mRNA expression also showed circadian rhythm having acrophase at around CT18 in the middle of *per1* and *cry1*, while *hes1* mRNA expression having the lowest value at CT18. Moreover, all of these rhythms disappeared in *per1*$^{-/-}$*per2*$^{-/-}$ mouse liver. These results suggest that *hes6* and *hes1* are clock controlled genes (ccgs) that may have roles in the mouse liver.

The *hes6* gene structure and conserved DNA elements

To investigate the molecular mechanism allowing circadian expression of *hes6*, mouse, rat and human *hes6* were analyzed and summarized in Figure 4A. The mouse and human *hes6* genes are composed of four exons and three introns, while rat *hes6* is composed of three exons and two introns. The *hes6* promoters up to 1.2 kb are well conserved among three species: Especially, Ngn1/3, NeuroD1, MyoD binding sites important for neurogenesis or myogenesis are placed forming clusters in two distinct locations of promoter region (about -1 kb or -0.2 kb region). This conforms to the findings that *hes6* is mainly regulated during neurogenesis and myogenesis (Bae *et al*., 2000; Murai *et al*., 2007). Interestingly, three E-boxes, putative binding sites for positive circadian transcription factor complex CLOCK/BMAL1 were found conserved: one on ~-0.9 kb, and the other two on inton 3 and exon IV. This raises the possibility that *hes6* may be regulated by CLOCK/BMAL1.

Regulation of *hes6* gene transcription by CLOCK/BMAL1

Several constructs were generated to examine the possible regulation of *hes6* gene transcription by CLOCK/BMAL1 (Figure 4B). The serial deletional constructs were made from mouse *hes6* promoter region and regulatory constructs were from intragenic sequence. In the upstream reporters, the longest construct, -1254/+65-Luc, has an E-box and in the regulatory constructs HireA-Luc and HireB-Luc have one E-box each and Hire AB-Luc has two downstream E-boxes. These constructs were transiently co-transfected with CLOCK/BMAL1 expression vectors into NIH 3T3 cells and HT22 cells (Figures 4C and 4D). The significant and reproducible induction by CLOCK/BMAL1 appeared only in HireB-Luc and HireAB-Luc constructs. This result indicates that the regulatory sequence containing exonic E-box is responsible for the induction by CLOCK/BMAL1.

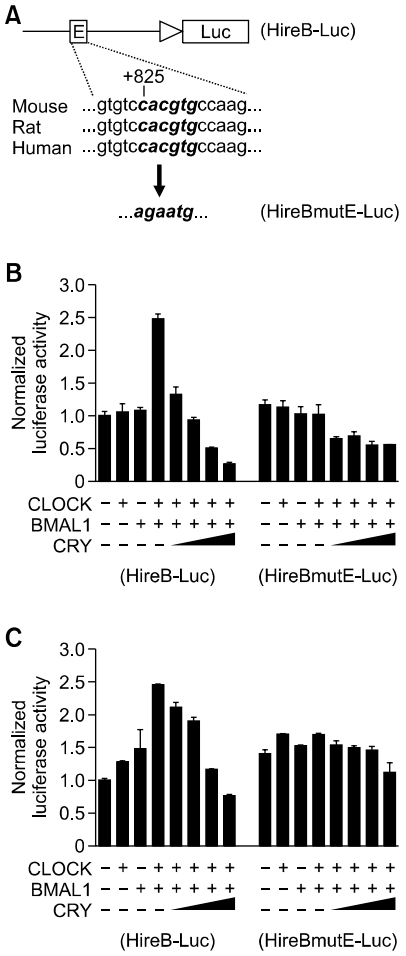

Figure 5. Conserved E-box in the exon IV of mouse *hes6* gene is responsible for induction by CLOCK/BMAL1 and inhibition by CRY1. (A) Location of conserved E-box in HireB-Luc construct and point mutation resulting in HireBmut-Luc construct. Large triangle indicates SV40 promoter. (B, C) Effect of CLOCK/BMAL1 and increasing amount of CRY1 expression on reporter activity. NIH 3T3 mouse fibroblasts (B) and HT22 mouse hippocampal cells (C) were transiently transfected with either HireB-Luc or HireBmutE-Luc construct with or without CLOCK/BMAL1 and CRY1 expression vectors. Resulting firefly luciferase activity were normalized to *Renilla* luciferase activity driven by TK promoter, which were used as an internal control. Data are expressed as mean ± S.E.M.

The functionality of exonic E-box

To confirm the functionality of E-box on exon IV, point mutation studies were performed. The E-box sequence of HireB-Luc, CACGTG, was mutated to

AGAATG (Figure 5) using HireB-Luc as a template. The WT and mutated constructs were transiently co-transfected with CLOCK/BMAL1 and with or without CRY expression vector into NIH 3T3 and HT22 cells (Figures 5B and 5C). The induction by CLOCK/BMAL1 and repression by CRY1 observed in the HireB-Luc disappeared in the HireBmutE-Luc construct. Chromatin immunoprecipitation also demonstrated the binding of BMAL1 to this region *in vivo* (data not shown). These results clearly indicate that E-box on exon IV is functional.

Discussion

The molecular clockwork plays important roles in diverse physiological and pathological settings by controlling timed expression of other regulatory and/or structural genes (reviewed in Takahashi *et al*., 2008). Especially notable is metabolic clockwork which has great implications in diverse human pathologies including metabolic syndrome (reviewed in Green *et al*., 2008). The present work demonstrating the role of CLOCK/BMAL1, Hes1 and Hes6 in the LDLR expression suggests multiple and complex regulation of LDLR at the transcription level.

The daily changes of LDLR protein level in the rat was reported previously (Balasubramaniam *et al*., 1994) and this work provides the possible mechanism underlying it. Using HepG2 human hepatocarcinoma cells, we demonstrated that CLOCK/BMAL1 induces *ldlr* transcription in a serum-independent manner (Figure 2; see also Figure S2). Accordingly, *ldlr* mRNA in the wild-type mouse liver peaked around CT12 (Figure 2) at the time other E-box regulated genes such as *per1* and *cry1* are also upregulated (Figure 3). This implies that LDLR is one of the clock-controlled genes and elevates LDLR expression before major mealtime in mice (Sanchez-Alavez *et al*., 2007; Yoon *et al*., 2012).

In the present study, Hes family members Hes1 and Hes6 downregulated human LDLR expression only under serum-depleted conditions in HepG2 cells (Figures 2B and 2C), although they downregulated mouse LDLR in a serum-independent fashion (Figure S2). This indicates that the upregulation of LDLR in the absence of serum or cholesterol (Jeong *et al*., 2009 and references therein) can be efficiently blocked by Hes1 and Hes6 whose expressions are also under the control of circadian clock and involve distinct promoter elements of LDLR. The convergent and divergent promoter structures and/or differential control of human and mouse *ldlr* genes (Figures S1 and S2) are quite intriguing and necessitate more extensive works.

LDLR is a cell-surface glycoprotein that serves in the homeostatic control of blood cholesterol by mediating the elimination of cholesterol-containing lipoprotein particles from blood circulation (Brown and Goldstein, 1986). In case of sterol depletion, the *ldlr* transcription markedly increases, while it decreases in case of sterol accumulation (Goldstein and Brown, 1990; Ellsworth *et al*., 1994). It is known that serum depletion or lipoprotein deficiency powerfully induces the LDLR expression in cultured HepG2 cells (Pak *et al*., 1996). Sterol-mediated repression or induction of *ldlr* promoter is mediated by sterol regulatory element-1 (SRE-1) (Smith *et al*., 1990), which has been shown to bind SRE-BP-1 (SRE-binding protein-1) and SRE-BP-2 (SRE-binding protein-2) (Briggs *et al*., 1993; Wang *et al*., 1993). In the absence of sterol, proteases cleave the SRE-BP, which allows translocation of the truncated SREBP to the nucleus where it activates transcription of the LDL receptor.

The present study showed that Hes1 exerts its effect through sterol regulatory element of the LDLR promoter (Figure 2) which is bound by SRE-BP when intracellular cholesterol level goes lower. Thus, the possible interference of Hes1 with SRE-BP action is an intriguing possibility that needs to be resolved in the future. In contrast, the action of Hes6 is independent of SRE but involves the similar proximal region of the LDLR promoter. So far, it was fruitless to delineate the regulatory element(s) that is responsible for it. As Hes6 is known to interfere with other transcription factors such as Hes1 and MyoR under different cellular context, an indirect action of Hes6 remains an open question.

The present work analyzed mouse, rat, and human *hes6* genes promoter and coding sequences and found that many transcription factor binding sites important for neurogenesis and myogenesis are well conserved as expected (Figure 4). Moreover, a fully functional E-box is found within the exon IV of the *hes6* gene as demonstrated by point mutation (Figure 5) and chromatin immunoprecipitation assay (data not shown). Accordingly, *hes6* gene expression oscillated in an anti-phasic manner with *hes1* in the mouse liver in a functional clock-dependent manner (Figure 3) and seems to be involved in the regulation of LDLR. Although aberrant expression of Notch signaling genes including Hes1 and Hes6 was reported in the hepatocellular carcinogenesis (Gramantieri *et al*., 2007), this is the first demonstration that Hes6 and Hes1 may have a functional role in the liver regulating cholesterol homeostasis.

The present work poses critical implications for

human metabolic diseases (Al Rayyes *et al*., 1997; Hazra *et al*., 2008). For instance, the human genetic disease called familial hypercholesterolemia (FH) is caused by genetic defects in the LDLR. The FH patients possess increased risk of high blood cholesterol and display myocardial infarctions (heart attacks), orange-yellow xanthomas, atherosclerosis, and cardiovascular diseases (Brown and Goldstein, 1986; Goldstein and Brown, 2009). Thus, the aberrant expression and dysregulation of LDLR in the absence of functional clock, *hes1*, or *hes6 in vivo* is an intriguing possibility that is under investigation.

In modern society, more and more workers are involved in various kinds of shift works. Epidemiological evidence indicates that shift works are closely associated with increased risk for metabolic syndrome (Szosland, 2010). Moreover, an animal model of circadian disturbance increased mortality in mice (Park *et al*., 2012). Low density lipoprotein receptor (LDLR) is known to play a key role in cholesterol metabolism that has been associated with an increased risk of coronary heart disease and atherosclerosis (Brown and Goldstein, 1986). Thus, together with our recent observation that meal time shift disturbs circadian rhythmicity and metabolic homeostasis in mice (Yoon *et al*., 2012), possible circadian regulation of LDLR promoter by CLOCK/BMAL1, Hes1 and Hes6 poses great implications for human health and mortality.

Methods

Chemicals and reagents

Unless indicated otherwise, all the chemicals and reagents were obtained from Sigma.

Animals, entrainment, and circadian sampling

All animal experiments were approved and performed under the guidelines of Kyung Hee University Institutional Animal Care and Use Committee. PDK mice have been described elsewhere (Park *et al*., 2012). All cages were placed and maintained in a light-proof Clean Animal Rack cabinet (Shin Biotech, Seoul, Korea) with constant air ventilation. Lighting schedule was automatically controlled by equipped digital timers and the ambient temperature was maintained at 23 $\pm$ 1°C. Light intensity during the light phase was maintained at 350-450 lux throughout the area. Adult wild-type and *per1*$^{-/-}$*per2*$^{-/-}$ male mice were entrained to a 12:12 LD photoperiodic cycle with light-on at 08:00 AM. After 2 weeks of entrainment, animals were released to constant darkness (DD). On the second day after light-off, animals were sacrificed and liver samples were obtained at the indicated circadian time.

Blood collection and measurements of glucose and cholesterol in the blood

8 week-old wild-type male mice were entrained to a 12:12 LD photoperiodic cycle for 2 weeks and sacrificed by cervical dislocation at the indicated zeitgeber time (ZT) and whole blood samples were collected into tubes containing heparin. Blood glucose levels were determined using Accu-Chek® Performa kit (Roche) at the time of sacrifice. Remaining blood samples were centrifuged at 3,000 rpm at 4°C for 10 min. Triglyceride, total cholesterol and HDL cholesterol levels in the upper plasma were determined using specific kits and CR-3000 apparatus from Callegrari™ according to the manufacturer's specifications (Yoon *et al*., 2012).

Promoter analysis

Genomic DNA sequences spanning exons, introns, and 3 Kb upstream promoter region of mouse (Accession No. NT078297, chromosome 1), rat (NW_047817, chromosome 9), and human *hes6* (NT_005120, chromosome 2) were accessed at NCBI website (http://www.ncbi.nlm.nih.gov). These sequences were analyzed for the common transcription factor binding sites using tools provided by Genomatrix (www.genomatrix.de). Conserved DNA elements were found and used as a reference for the generation of reporter constructs.

Cloning of reporter constructs

The LDLR reporter constructs were described elsewhere (Jeong *et al*., 2009). The serial deletion reporter constructs (-1254, -963, -187, -111/+65m*hes6*-Luc) and intronic regulatory element reporter constructs (HireA, HireB, HireAB-Luc) of *hes6* were generated by PCR with each set of primers with KpnI or NheI linker as diagramed in Figure 4. PCR products were cloned into the pGL3-basic or pGL3-promoter vector (Promega) at KpnI/NheI site. Correct cloning was verified by restriction enzyme cutting and subsequent DNA sequencing. Primer sequences used are as follows: H6up1(KpnI linker): atg gta cca aat cca aga acg ctg gag c; H6up2 (KpnI linker): atg gta cca agt tac tgg ggc cag aca g; H6up3 (KpnI linker): atg gta cct aag tgg cag gag gtc tgg c; H6up4 (KpnI linker): atg gta ccc tct cat tgg cta cag gct g; H6dn5 (NheI linker): atg cta gcc tag tgc aaa cta ctc agc g; HireAup (KpnI linker): atg gta cct ctg cgc agg tgc agg cca a; HireBdn (NheI linker): atg cta gca agc cct tgc cat gga tag g; HireCup (KpnI linker): atg gta cct tcc cac cct cct gca cag; HireDdn (NheI linker): atg cta gct ctg gga tct cct cta ggt c.

Cell culture, transient transfection, and dual luciferase reporter assay

HT22 mouse hippocampal cells, NIH 3T3 mouse embryonic fibroblast cells, and HepG2 human hepatocarcinoma cells were cultivated in DMEM supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C (Doi *et al*., 2007; Park and Pak, 2011). For transient transfection, cells were seeded in 24-well plates at a density of 5×10^4 per well. On the following day, cells were trans-

fected with each of the luciferase reporter construct and expression vectors using lipofectamine reagent (Invitrogen). To compensate transfection efficiency, 20 ng of the minimal thymidine kinase promoter-driven *Renilla* luciferase construct (pRL-mTK) was cotransfected. The total amount of plasmid was held constant by adding pcDNA3 plasmid as a carrier DNA. The plasmids used for transfection were prepared using QIAGEN miniprep kit. After 48 h of transfection, cells were lysed and luciferase activity was measured using dual lucifease reporter assay kit (Promga) and DLReady Berthold luminometer (Centro LB960) according to the manufacturer's specifications. The firefly luciferase activity was normalized to *Renilla* luciferase activity and presented as relative values of the control transfection.

Point mutation of reporter constructs

in vitro mutagenesis of conserved E-box to generate HireBmutE-Luc (CACGTG → AGAATG) was performed on the HireB-Luc construct using Quick Change Site-Directed Mutagenesis kit (Stratagene). The primer set for mutated E-box is 5'-CAC ACG TTC GTG TCA GAA TGC CAA GCC ATC GAT G and 5'-CAT CGA TGG CTT GGC ATT CTG ACA CGA ACG TGT G.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated from tissues and cells using acid guanidinium phenol-chloroform (AGPC) method (Cho *et al*., 1998). One μg of total RNA was reverse transcribed using RTase M-MLV (Takara, 2640A) with 200 ng of random hexamer, 2.5 mM each of dNTP and RNase inhibitor (Takara, 2310A) at 37°C for 1 h. The procedure for real-time PCR using LightCycler has been described elsewhere (Doi *et al*., 2007). Briefly, the standard was prepared by pooling 5-fold diluted RT samples in 1mM Tris. The templates for real-time PCR were prepared by further 15-fold dilution of 5-fold diluted RT samples. 2X SYBR Premix EX Taq (Takara, RR041A) and LightCycler Version 1.5 (Roche, Salt Lake City) were used for the real-time PCR. The expression levels of *gapdh* were used for normalization. Primer sequences used for real-time PCR are summarized in Supplemental Data Table S1.

Supplemental data

Supplemental data include two figures and a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-11-02.pdf.

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